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(21) International Application Number: PCT/US92/06869 (22) International Filing Date: 14 August 1992 (14.08.92) (30) Priority data: 745,591 15 August 1991 (15.08.91) US (60) Parent Application or Grant (63) Related by Continuation US 745,591 (CIP) Filed on 15 August 1991 (15.08.91) (71) Applicant (for all designated States except US): BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only) : HANSEN, Eric, J. [US/US]; 2404 Chamberlain, Plano, TX 75023 (US). HELMINEN, Merja [FI/US]; 6031 Pineland # 1116, Dallas, TX 75231 (US). MACIVER, Isobel [GB/US]; 6721 Larmanda, Suite 254, Dallas, TX 75231 (US). (74) Agent: PARKER, David, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US). (81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: METHODS AND COMPOSITIONS RELATING TO USEFUL ANTIGENS OF <i>MORAXELLA CATARRHALIS</i> (57) Abstract The present disclosure relates to selected antigenic proteins obtained from the outer membranes of <i>Moraxella catarrhalis</i> , that are found to have a variety of useful properties. These proteins, termed OMPs ("Outer Membrane Proteins"), are characterized as having molecular weights of about 30 kD, 80kD and between about 200 and 700 kD, respectively. Studies set forth herein demonstrate that monoclonal antibodies directed against these proteins confer a protective effect against infection by <i>Moraxella catarrhalis</i> organisms in animal models, demonstrating the potential usefulness of such antibodies in conferring passive immunity as well as the potential usefulness of these OMPs, or variants thereof, in the preparation of vaccines. Also disclosed are DNA segments encoding these OMPs, methods for preparing the antigens, or variants, through the application of recombinant DNA techniques, as well as diagnostic methods and embodiments.		

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DescriptionMETHODS AND COMPOSITIONS RELATING TO
USEFUL ANTIGENS OF MORAXELLA CATARRHALIS

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BACKGROUND OF THE INVENTION1. Field of the Invention

10 The present invention relates generally to various
outer membrane proteins (OMPs) of *Moraxella catarrhalis*
which have been found by the inventors to be useful
targets in immunotherapy, such as in the preparation of
vaccines or protective antibodies for use in treatment of
15 *Moraxella catarrhalis*-related diseases. In particular
aspects, the present invention concerns antigens
identified by molecular weights of about 30 kD, 80 kD and
a third antigen, termed "high molecular weight protein"
or "HMWP" antigen having a molecular weight of between
20 about 200 and 700 kD, as measured by SDS-polyacrylamide
gel electrophoresis. In other aspects, the invention
concerns recombinant clones encoding these antigens,
antigen fragments derived therefrom, equivalents thereof,
as well as to antibodies reactive with these species.
25 Further, the invention concerns methods for the detection
of *Moraxella catarrhalis* antigens and antibodies, as well
as the use of specific antigens and antibodies both in
passive and active immunity against *Moraxella catarrhalis*
infections.

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2. Description of the Related Art

It was previously thought that *Moraxella catarrhalis*
(previously known as *Branhamella catarrhalis* or *Neisseria*
35 *catarrhalis*) was a harmless saprophyte of the upper
respiratory tract. However, during the previous decade,
it has been determined that this organism is an important

human pathogen. In fact, recent studies have established this Gram-negative diplococcus as the cause of a number of human infections (Murphy, 1989). For example, *Moraxella catarrhalis* is a leading cause of otitis media, acute maxillary sinusitis as well as generalized infections of the lower respiratory tract (see, e.g., Murphy et al., 1989). Studies have established that the incidence of otitis media and sinusitis attributed to *Moraxella catarrhalis* infections is increasing, with it being about the third most common causative organism. In fact, reports have identified otitis media as the most common disease for which infants and children receive health care (Consensus, 1989).

The "Consensus" report referred to above concluded that prevention of otitis media is an important health care goal due to both its occurrence in infants and children, as well as certain populations of all age groups. In fact, the total financial burden of otitis media has been estimated to be at least 2.5 billion annually, or approximately 3% of the health care budget. Vaccines were identified as the most desired approach to the prevention of this disease for a number of reasons. For example, it was estimated that if vaccines could reduce the incidence of otitis media by 30%, this outcome could bring about an annual health care savings of at least \$400 million. However, while some progress has been made in the development of vaccines for 2 of the 3 common otitis media pathogens, *Streptococcus pneumoniae* and *Haemophilus influenzae*, there is no indication that similar progress has been made with respect to *Moraxella catarrhalis*. This is particularly troublesome in that *Moraxella catarrhalis* now accounts for approximately 17-20% of all otitis media infection (Murphy, 1989).

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Previous attempts have been made to identify and characterize *Moraxella catarrhalis* antigens that would

serve as potentially important targets of the human immune response to infection (Murphy, 1989; Goldblatt et al., 1990; Murphy et al., 1990). Generally speaking, the surface of *Moraxella catarrhalis* is composed of outer
5 membrane proteins (OMPs), lipooligo-saccharide (LOS) and fimbriae. As Murphy points out, *Moraxella catarrhalis* appears to be somewhat distinct from other gram-negative bacteria in that attempts to isolate the outer membrane
10 of this organism using detergent fractionation of cell envelopes has generally proven to be unsuccessful in that the procedures did not yield consistent results. Moreover, preparations were found to be contaminated with cytoplasmic membranes which suggest an unusually
15 characteristic of the *Moraxella catarrhalis* cell envelope.

However, workers in the field have demonstrated the existence of 7 or 8 major OMP species, and these appear to be fairly consistent from *Moraxella catarrhalis* strain
20 to strain, in spite of the great diversity of stains tested. For example, Campagnari et al. has identified the OMPs by letters A-H beginning with a band of molecular weight 98 Kd (OMP-A) and proceeding to the band with a molecular weight of about 21 Kd (OMP-A).
25 (Campagnari et al., 1987).

The LOS of *Moraxella catarrhalis* has also been suggested as a possible target for vaccine development. LOS has been isolated from *Moraxella catarrhalis* strains
30 and subjected to SDS-PAGE and silver staining (Murphy, 1989). It was reported that all but one strain produced an identical pattern of LOS staining. Thus, it appears that the LOS of *Moraxella catarrhalis* is very highly antigenically conserved, thus raising the feasibility of
35 using a portion of the LOS molecule as a vaccine component.

Lastly, the Fimbriae have been suggested as a possible vaccine candidate. Fimbriae apparently play a role in adherence and colonization of mucosal services in some bacteria. Workers in the field have postulated that if antigenically conserved epitopes are expressed on fimbriae and can be identified, then it is possible that antibodies to such epitopes might be useful therapeutically, or that such epitopes can serve as vaccine components.

Unfortunately, although various subcomponents of the *Moraxella catarrhalis* cell have been suggested as places to begin a search for vaccine candidates, there has still been no such candidate identified. Certainly, no antigenic epitope or epitopes have been shown to induce protective antibodies. Thus, it is clear that there is currently a need to identify which, if any, *Moraxella catarrhalis* component may serve as useful antigens that can, for example, be employed in the preparation of both passive and active immunotherapeutic reagents such as vaccines. Additionally, once such an antigen or antigens is identified, there is a need for providing methods and compositions which will allow the preparation of these vaccines and quantities that will allow their use on a wide scale basis in therapeutic protocols.

SUMMARY OF THE INVENTION

Accordingly, in a general and overall sense, the present invention is concerned with the identification and subsequent preparation of an *Moraxella catarrhalis* antigen species that would be of use both in the prevention and diagnosis of disease. In more particular terms, the invention concerns the inventors' surprising discovery that particular *Moraxella catarrhalis* OMP antigens, including the 30 kD, 80 kD and HMWP OMP antigens, have particular utility in vaccine development.

5 It is postulated by the inventors, therefore, these antigens can be used directly as a component of a vaccine, or can be employed for the preparation of corresponding or equivalent antigen through sequence analysis.

10 It should be pointed out that of these OMP antigens, the inventors believe that the 30 kd and HMWP species will prove to be the most useful, in that their studies have shown that antibodies directed against these two OMP species are broadly reactive with *Moraxella catarrhalis* subtypes and isolates. However, antibodies against the 80 kd species have not, as yet, been shown to react with all subspecies, and thus may not be pan-reactive. Thus, 15 particularly preferred embodiments of the invention concern the 30 kd and HMWP OMP antigens, DNA fragments encoding these antigens and related species, antibodies recognizing these antigen species, and the like.

20 In certain embodiments, the present invention thus concerns an antigen composition comprising a purified protein or peptide antigen incorporating an epitope that is immunologically cross-reactive with one or more of the foregoing *M. catarrhalis* OMP antigens. While, generally, 25 the purified protein or peptide antigen will comprise the OMP itself, the present disclosure provides techniques which may be employed for preparing variants of these OMP antigens, peptides that incorporate related antigenic epitopes, as well as antigenic functional equivalents of 30 each of these. Furthermore, in that DNA segments encoding the various OMP antigens are disclosed, the antigens may be provided essentially free of antigenic epitopes from other *M. catarrhalis* antigens through the application of recombinant technology. That is, one may 35 prepare the antigen by recombinant expression means using a host cell other than *M. catarrhalis* or related species, and thereby provide the antigen in an essentially pure

antigenic state, with respect to other *M. catarrhalis* antigens. Such preparations will therefore be free, e.g., of LOS or fimbriae antigens.

5 In still further embodiments, through the use of standard DNA sequencing technology, DNA segments disclosed herein may be sequenced, and from this DNA sequence one may determine the underlying amino acid sequence of the selected OMP protein, whether it be the
10 30 kD, 80 kD or HMWP OMP species. Once this information is obtained, identification of suitable antigenic epitopes is a relatively straightforward matter through the use of, for example, software programs for the prediction of such epitopes that are available to those
15 of skill in the art. The amino acid sequence of these "epitopic core sequences" may then be readily incorporated into shorter peptides, either through the application of peptide synthesis or recombinant technology.

20 Preferred peptides will generally be on the order of 15 to 50 amino acids in length, and more preferably about 15 to about 30 amino acids in length. It is proposed that shorter antigenic peptides which incorporate
25 epitopes of the selected OMP will provide advantages in certain circumstances, for example, in the preparation of vaccines or in immunologic detection assays. Exemplary advantages include the ability to circumvent problems of contamination and purity often associated with proteins
30 prepared by recombinant production in that peptides of this length may be prepared readily by synthetic means using peptide synthesizers.

35 In other embodiments, the present invention concerns processes for preparing compositions which include purified protein or peptide antigens that incorporate epitopes that are immunologically cross-reactive with the

30 kD, 80 kD or HMWP OMP. In a general sense, these processes include first selecting cells that are capable of expressing such a protein or peptide antigen, culturing the cells under conditions effective to allow expression of the antigen, and collecting the antigen to thereby prepare the composition. Where one desires to prepare the OMP antigen itself, one will simply desire to culture *M. catarrhalis* cells as a first step. In this case, the antigen will be provided, upon expression, in the outer membrane fraction of the cell. The antigen is then prepared by, first, preparation of membrane fraction followed by solubilization and extraction of the antigen from the prepared membranes using an ionic or non-ionic detergent. Further purification may be achieved by a variety of methods including column fractionation, isoelectric focusing, and the like, or even immunoadsorption employing OMP-directed antibodies.

Of course, in light of the disclosure herein one may choose more preferred embodiments to prepare the desired antigen that include expressing a recombinant DNA segment encoding the antigen in a recombinant host cell. Preferred recombinant host cells for expression of antigens in accordance with the invention will typically be a bacterial host cell in that the antigen is a bacterial antigen. Preferred bacterial host cells include *E. coli*, *H. influenzae*, *Salmonella* species, *Mycobacterium* species, or even *Bacillus subtilis* cells. Of course, where desired, one may also express the desired antigen or antigens in eukaryotic cells.

As indicated above, in particular embodiments, the present invention concerns DNA segments which encode the desired protein or peptide antigen. Methods are disclosed herein for obtaining such segments in a purified state relative to their naturally occurring state. These DNA segments will have a number of advantages and uses. For

example, segments encoding the entire OMP gene may be introduced into recombinant host cells and employed for expressing the entire protein antigen. Alternatively, through the application of genetic engineering techniques, subportions or derivatives of the selected OMP gene may be employed to prepare shorter peptide sequences which nevertheless incorporate the desired antigenic epitopes. Furthermore, through the application of site-directed mutagenesis techniques, one may re-engineer DNA segments of the present invention to alter the coding sequence, e.g., to introduce improvements to the antigenicity of epitopic core sequences and thereby prepare antigenically functional equivalent peptides. Of course, where desired, one may also prepare fusion peptides, e.g., where the antigen coding regions are aligned within the same expression unit with other desired antigen or proteins or peptides having desired functions, such as for immunodetection purposes (e.g., enzyme label coding regions).

Depending on the host system employed, one may find particular advantages where DNA segments of the present invention are incorporated into appropriate vector sequences which may, e.g., improve the efficiency of transfection of host cells. Where bacterial host cells are employed, it is proposed that virtually any vector known in the art to be appropriate for the selected host cell may be employed. Thus, in the case of *E. coli*, one may find particular advantages through the use of plasmid vectors such as pBR322, or bacteriophages such as λ GEM-11. Other particular examples are disclosed hereinbelow.

In the preparation of recombinant clone banks from which appropriately transfected cells are selected, it will generally be the case that expression of the selected OMP gene sequences can be achieved in such host cells without the use of vectors having their own

intrinsic promoter sequences. This is because the genomic *M. catarrhalis* DNA fragments employed for clone bank preparation will include endogenous promoters associated with the various coding sequences. However, 5 the inventors propose that one may ultimately desire to re-engineer the promoter region of the antigen-coding fragments of the present invention to introduce heterologous promoter. This may allow one to overexpress the OMP antigen in relation to its natural expression by 10 *M. catarrhalis* cells.

It is contemplated that nucleic acid segments of the present invention will have numerous uses other than in connection with expression of antigenic peptides or 15 proteins. For example, nucleic acid segments of at least 14 or so nucleotides in length that incorporate regions of the OMP gene sequence may be employed as selective hybridization probes for the detection of *M. catarrhalis* sequences in selected samples or, e.g., to screen clone 20 banks to identify clones which comprise corresponding or related sequences. Furthermore, short segments may be employed as nucleic acid primers, such as in connection with PCR technology, for use in any of a number of applications, including, e.g., cloning and engineering 25 exercises, or in PCR-based detection protocols.

In still further embodiments, the invention concerns the preparation of antibodies capable of immunocomplexing with epitopes of the OMP antigen. Particular techniques 30 for preparing antibodies in accordance with the invention are disclosed hereinbelow. However, it is proposed by the inventors that any of the current techniques known in the art for the preparation of antibodies in general may be employed, through the application of either monoclonal 35 or polyclonal technology. As noted above, a surprising aspect of the invention involves the inventors' discovery that monoclonal antibodies directed against the 30 kD, 80

kD and HMWP OMP antigens provide a protective effect against *M. catarrhalis* challenge in animal models. This surprising finding indicates not only that antibodies may be employed in the preparation of compositions for use in connection with passive immunotherapy, but further, that epitopes of these OMP antigens may be employed in the preparation of vaccine compositions. Accordingly, the present invention is directed both to vaccine compositions which include an antigen in accordance with the present invention, or antibodies against such an antigen, together with a pharmaceutically acceptable carrier, diluent, or adjuvant.

In still further embodiments, the present invention concerns immunodetection methods and associated kits. It is proposed that antigens of the present invention may be employed to detect antibodies having reactivity therewith, or, alternatively, antibodies prepared in accordance with the present invention, may be employed to detect antigens. In general, these methods will include first obtaining a sample suspected of containing such an antigen or antibody, contacting the sample with an antibody or antigen in accordance with the present invention, as the case may be, under conditions effective to allow the antibody to form an immunocomplex with the antigen or antibody to be detected, and detecting the presence of the antigen in the sample by detecting the formation of an immunocomplex.

In general, the detection of immunocomplex formation is quite well known in the art and may be achieved through the application of numerous approaches. For example, the present invention contemplates the application of ELISA, RIA, immunoblot, dotblot, indirect immunofluorescence techniques and the like. Generally, immunocomplex formation will be detected through the use of a label, such as a radiolabel or an enzyme tag (such

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as alkaline phosphatase, horseradish peroxidase, or the like). Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

For diagnostic purposes, it is proposed that virtually any sample suspected of comprising either the antigen or antibody sought to be detected, as the case may be, may be employed. Exemplary samples include clinical samples obtained from a patient such as blood or serum samples, ear swabs, sputum samples, middle ear fluid or even perhaps urine samples may be employed. Furthermore, it is contemplated that such embodiments may have application to non-clinical samples, such as in the titering of antigen or antibody samples, in the selection of hybridomas, and the like.

In related embodiments, the present invention contemplates the preparation of kits that may be employed to detect the presence of antigens and/or antibodies in a sample. Generally speaking, kits in accordance with the present invention will include a suitable OMP antigen (i.e., either the 30 kD, 80 kD or HMWP species, or protein containing epitopes corresponding to one or more of these species), or antibody directed against such an antigen, together with an immunodetection reagent and a means for containing the antibody or antigen and reagent. The immunodetection reagent will typically comprise a label associated with the antibody or antigen, or associated with a secondary binding ligand. Exemplary ligands might include a secondary antibody directed against the first antibody or antigen or a biotin or avidin (or streptavidin) ligand having an associated label. Of course, as noted above, a number of exemplary labels are known in the art and all such labels may be employed in connection with the present invention.

The container means will generally include a vial into which the antibody, antigen or detection reagent may be placed, and preferably suitably aliquoted. The kits
5 of the present invention will also typically include a means for containing the antibody, antigen, and reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are
10 retained.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Western blot analysis of *M. catarrhalis*
15 proteins using as a probe monoclonal antibody 10F3, which recognizes the 80 kD OMP. Lane A is a Rainbow protein molecular weight marker (M.W. 14.3 to 200 kD, Amersham); Lane B is a negative control comprising a whole cell lysate of 4B1/pBR322/RR1 (4B1 is an *M. catarrhalis* gene
20 encoding an unrelated protein recognized by monoclonal antibody 4B1); Lanes C and D are whole cell lysates of 10F3/pBR322/RR1; and Lane E is a blank control.

25 Figure 2. Preliminary restriction map of pMEH120, which comprises a segment encoding the Mab 10F3-reactive 80 kD antigen.

30 Figure 3. Preliminary restriction map of phage MEH200, which comprises a segment encoding the Mab 17C7-reactive HMWP antigen.

35 Figure 4. Western blot analysis of *M. catarrhalis* proteins using as a probe monoclonal antibody 8B6, which recognizes the 30 kD OMP. Lane A is a Rainbow protein molecular weight marker (M.W. 14.3 to 200 kD, Amersham); Lane B is a prestained SDS-PAGE-standard, low molecular

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weight (M.W. 16 to 110 kD, Bio-Rad); Lane C contains proteins from a phage lysate of recombinant *E. coli* that express the 30 kD OMP (LE392/8B6); Lane D is a blank control; Lane E is a negative control (phage lysate from recombinant *E. coli* expressing the HMWP OMP, LE392/17C7); and Lane F is a positive control (*M. catarrhalis* 035E outer membrane vesicles).

Figure 5. Western blot analysis of *M. catarrhalis* proteins using as a probe monoclonal antibody 17C7, which recognizes the HMWP OMP. Lane A is a Rainbow protein molecular weight marker (M.W. 14.3 to 200 kD, Amersham); Lane B is a prestained SDS-PAGE-standard, low molecular weight (M.W. 16 to 110 kD, Bio-Rad); Lanes C, D and E contain proteins from a phage lysate of recombinant *E. coli* that express the HMWP OMP (LE392/17C7); Lane F is a blank control; Lane H is a negative control (phage lysate from recombinant *E. coli* expressing the 30 kD OMP, *E. coli*/8B6 phage lysate); and Lane G is a positive control (*M. catarrhalis* 035E outer membrane vesicles).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to the inventors' identification of particular outer membrane proteins (OMPs) of *Moraxella catarrhalis* that are found to have particularly useful properties, e.g., in the preparation of both diagnostic and therapeutic reagents. These proteins appear to be cell surface-exposed in their natural state, and exhibit molecular weights of about 30 kD, 80 kD and between about 200 and 700 kD, respectively, upon SDS-PAGE. Particular embodiments relate to the recombinant cloning of sequences encoding these proteins, antigenic subfragments, variants, and the like. The present invention also relates to monoclonal antibodies to these *M. catarrhalis* OMPs that are shown to reduce the number of infecting *M. catarrhalis* bacteria present in

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localized lung infections, as demonstrated in pulmonary clearance studies using a murine model system.

Recombinant clones, expressing one or more of the selected OMPs, and that may be used to prepare purified OMP antigens as well as mutant or variant protein species in significant quantities, are included within the scope of the disclosure. The selected OMP antigen, and variants thereof, are anticipated to have significant utility in diagnosing and treating *M. catarrhalis* infections. For example, it is proposed that these OMP antigens, or peptide variants, may be used in immunoassays to detect *M. catarrhalis* or as a vaccine to treat *M. catarrhalis* infections.

To assist those of skill in carrying out more particular aspects of the invention, recombinant clones bearing DNA segments encoding, respectively, the 30 kD, 80 kD and HMWP OMP antigens, were deposited with the American Type Culture Collection (ATCC) on August 4, 1992, under the provisions of the Budapest Treaty. In particular, plasmid pMEH300 (ATCC accession number 69049) bearing a segment encoding the 30 kD OMP antigen; plasmid pMEH 120 (ATCC accession number 75285) bearing a segment encoding the 80 kD OMP antigen; and phage MEH 200 (ATCC accession number 75286) bearing a segment encoding the HMWP antigen, were deposited in the form of either phage lysate (MEH 200), purified plasmid DNA (pMEH120) or recombinant *E. coli*, strain RR1 (pMEH300).

The pMEH300 plasmid can be characterized as a modified pLG338 vector in which pLG338 was digested with *Xho*I, and *Sac*I linkers added. This new vector contains a *Moraxella catarrhalis* chromosomal DNA insert of about 20 kb in size that can be excised by digestion with *Sac*I. This insert contains an *M. catarrhalis* gene encoding the 30 kD antigen reactive with monoclonal antibody 8B6. The

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total vector size is therefore approximately 27 kb, with the vector comprising only about 7.3 kb.

The gene encoding the 80 kD OMP was originally cloned in a pBR322-based recombinant plasmid, designated pMEH100. Subsequently, this gene was subcloned in pBluescript for sequencing analysis. This new plasmid, designated pMEH120, is what was deposited with the ATCC. Recombinant plasmid pMEH120 is a pBluescript II SK+ vector containing an insert of *M. catarrhalis* chromosomal NA approximately 4.5 kb in size, and encodes a protein of about 80 kD that is reactive with monoclonal antibody 10F3. A preliminary restriction map of pMEH120 is set forth in Figure 2.

The gene encoding the HMWP OMP antigen, reactive with Mab 17C7, was not subcloned out of the λ GEM-11 phage used for the cloning work described in the examples below, phage MEH200. The λ GEM-11 phage vector includes an *M. catarrhalis* chromosomal DNA insert of about 11 kb in size, which can be excised from the phage DNA by digestion with either *Sfi*I or *Sac*I. A preliminary restriction map is shown in Figure 3.

As will be appreciated by those of skill in the art in light of the detailed disclosure set forth herein, that the invention is in no way intended to be limited by the foregoing or other specific embodiments that were deposited with the ATCC.

The nucleic acid sequences which encode for the selected OMP antigen, or their variants, may be useful in hybridization or polymerase chain reaction (PCR) methodology to detect *M. catarrhalis*. Accordingly, included in the present invention disclosure is information which may be used to prepare a wide variety of DNA fragments having a number of potential utilities,

such as the preparation of relatively short immunogenic/antigenic peptidyl subfragments of the antigen, the use of DNA or RNA sequences in PCR and hybridization studies as probes for in vitro detection, as well as other useful medical and biomedical applications related to the research, diagnosis and treatment of *M. catarrhalis* infections.

The OMP antigens of the present invention are referred to, respectively, as the 30 kD, 80 kD and HMWP OMPs. These proteins have been identified by the inventors by reference to monoclonal antibodies that were selected from a battery of monoclonal antibodies against *M. catarrhalis* outer membrane vesicles. These antibodies were employed as Western blot probes to identify corresponding antigens from SDS-PAGE runs of *M. catarrhalis* 035E outer membrane vesicle preparations. The monoclonal antibody recognizing the 30 kD OMP is termed 8B6, the antibody recognizing the 80 kD OMP is termed 10F3, and that recognizing the HMWP kD antigen has been designated 17C7 (see Figures 1, 4 and 5). Importantly, each of the foregoing hybridomas have been shown to be protective against *M. catarrhalis* infection in animal models.

As with the ATCC deposit of recombinant vectors and clones, hybridomas secreting the foregoing monoclonal antibodies that recognize the preferred OMP antigens have also been deposited with the ATCC under the provisions of the Budapest treaty on July 30, 1992. The deposited hybridomas secrete, respectively, monoclonal antibody 8B6 (ATCC accession number HB11091), which recognizes the 30 kD OMP antigen; monoclonal antibody 10F3 (ATCC accession number HB11092), which recognizes the 80 kD OMP antigen; and monoclonal antibody 17C7 (ATCC accession number HB11093) which recognizes the HMWP OMP antigen.

The present invention envisions various means for both producing and isolating the OMP antigen proteins of the present invention, ranging from isolation of purified or partially purified protein from natural sources (e.g.,
5 from *M. catarrhalis* bacterial cells), or from recombinant DNA sources (e.g., *E. coli* or microbial cells). In the latter case, the OMP antigens of the invention, or antigenic peptides derived therefrom, may be provided in essentially antigenically pure states in that they will
10 be free of other *M. catarrhalis* epitopes unrelated to the selected OMP species.

It is proposed that isolation of the OMP antigen from either natural or recombinant sources in accordance
15 with the invention may be achieved isolating cell envelopes or outer membranes and then using a detergent-based purification scheme. In the case of recombinant cells, the desired antigen may be present in inclusion bodies.

20 Since monoclonal antibodies to the 30 kD, 80 kD and HMWP OMP antigens are disclosed by the present invention, the use of immunoabsorbent techniques are anticipated to be useful in purifying the OMP antigen, or its
25 immunologically cross reactive variants. It is proposed that useful antibodies for this purpose may be prepared generally by the techniques disclosed hereinbelow, or as in generally known in the art for the preparation of monoclonals (see, e.g., U.S. Patents 4,514,498 and
30 4,740,467), and those reactive with the desired OMP protein or peptides selected. Moreover, it is believed that the foregoing general isolation scheme will work equally well for isolation of OMP variants or of antigenic/immunogenic subfragments of the protein,
35 requiring only the generation and use of antibodies having affinity for the desired peptidyl region.

Additionally, by application of techniques such as DNA mutagenesis, the present invention allows the ready preparation of so-called "second generation" molecules having modified or simplified protein structures. Second generation proteins will typically share one or more properties in common with the full-length antigen, such as a particular antigenic/immunogenic epitopic core sequence. Epitopic sequences can be provided on relatively short molecules prepared from knowledge of the peptide, or underlying DNA sequence information. Such variant molecules may not only be derived from selected immunogenic/ antigenic regions of the protein structure, but may additionally, or alternatively, include one or more functionally equivalent amino acids selected on the basis of similarities or even differences with respect to the natural sequence.

Epitopic Core Sequences of the OMP Antigens

As noted above, it is proposed that particular advantages may be realized through the preparation of synthetic peptides which include epitopic/immunogenic core sequences. These epitopic core sequences are identified herein in particular aspects as hydrophilic regions of the OMP antigen. It is proposed that these regions represent those which are most likely to promote T-cell or B-cell stimulation, and, hence, elicit specific antibody production. An epitopic core sequence, as used herein, is a relatively short stretch of amino acids that is "complementary" to, and therefore will bind, antigen binding sites on OMP-directed antibodies. Additionally or alternatively, an epitopic core sequence is one that will elicit antibodies that are cross-reactive with OMP directed antibodies. It will be understood that in the context of the present disclosure, the term "complementary" refers to amino acids or peptides that exhibit an attractive force towards each other. Thus,

certain epitope core sequences of the present invention may be operationally defined in terms of their ability to compete with or perhaps displace the binding of the desired OMP antigen with the corresponding OMP-directed antisera.

In general, the size of the polypeptide antigen is not believed to be particularly crucial, so long as it is at least large enough to carry the identified core sequence or sequences. The smallest useful core sequence anticipated by the present disclosure would be on the order of about 15 amino acids in length. Thus, this size will generally correspond to the smallest peptide antigens prepared in accordance with the invention. However, the size of the antigen may be larger where desired, so long as it contains a basic epitopic core sequence.

Accordingly, through the use of computerized peptide sequence analysis program (DNASTar Software, DNASTar, Inc., Madison, Wisc.), the inventor proposes to identify particular hydrophilic peptidyl regions of the 30 kD, 80 kD or HMWP OMP antigen which are believed to constitute epitopic core sequences comprising particular epitopes of the protein.

Syntheses of epitopic sequences, or peptides which include an antigenic epitope within their sequence, are readily achieved using conventional synthetic techniques such as the solid phase method (e.g., through the use of commercially available peptide synthesizer such as an Applied Biosystems Model 430A Peptide Synthesizer). Peptide antigens synthesized in this manner may then be aliquoted in predetermined amounts and stored in conventional manners, such as in aqueous solutions or, even more preferably, in a powder or lyophilized state pending use.

In general, due to the relative stability of peptides, they may be readily stored in aqueous solutions for fairly long periods of time if desired, e.g., up to
5 six months or more, in virtually any aqueous solution without appreciable degradation or loss of antigenic activity. However, where extended aqueous storage is contemplated it will generally be desirable to include
10 agents including buffers such as Tris or phosphate buffers to maintain a pH of 7.0 to 7.5. Moreover, it may be desirable to include agents which will inhibit microbial growth, such as sodium azide or Merthiolate. For extended storage in an aqueous state it will be
15 desirable to store the solutions at 4°C, or more preferably, frozen. Of course, where the peptide(s) are stored in a lyophilized or powdered state, they may be stored virtually indefinitely, e.g., in metered aliquots that may be rehydrated with a predetermined amount of water (preferably distilled) or buffer prior to use.

20

Antigenically Functional Equivalent Amino Acids

As noted above, it is believed that numerous modifications and changes may be made in the structure of
25 the desired OMP antigen, or antigenic/immunogenic subportions thereof, and still obtain a molecule having like or otherwise desirable characteristics.

It is, for example, known that certain amino acids
30 may be substituted for other amino acids in a protein structure in order to modify or improve its antigenic or immunogenic activity (see, e.g., Kyte et al, or Hopp, U.S. patent 4,554,101, incorporated herein by reference). For example, through the substitution of alternative
35 amino acids, small conformational changes may be conferred upon an antigenic peptide which result in increase affinity between the antigen and the antibody

binding regions. Alternatively, amino acid substitutions in certain OMP antigenic peptides may be utilized to provide residues which may then be linked to other molecules to provide peptide-molecule conjugates which
5 retain enough antigenicity of the starting peptide to be useful for other purposes. For example, a selected OMP peptide bound to a solid support might be constructed which would have particular advantages in diagnostic
10 embodiments.

The importance of the hydropathic index of amino acids in conferring interactive biologic function on a protein has been discussed generally by Kyte et al. (1982), wherein it is found that certain amino acids may
15 be substituted for other amino acids having a similar hydropathic index or core and still retain a similar biological activity. As displayed in the table below, amino acids are assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics. It
20 is believed that the relative hydropathic character of the amino acid determines the secondary structure of the resultant protein, which in turn defines the interaction of the protein with substrate molecules. Preferred
25 substitutions for monitoring binding capability will generally involve amino acids having index scores within ± 2 units of one another, and more preferably within ± 1 unit, and even more preferably, within ± 0.5 units.

TABLE I

	<u>Amino Acid</u>	<u>Hydropathic Index</u>
5	Isoleucine	4.5
	Valine	4.2
	Leucine	3.8
	Phenylalanine	2.8
	Cysteine/cystine	2.5
10	Methionine	1.9
	Alanine	1.8
	Glycine	-0.4
	Threonine	-0.7
	Tryptophan	-0.9
15	Serine	-0.8
	Tyrosine	-1.3
	Proline	-1.6
	Histidine	-3.2
	Glutamic Acid	-3.5
20	Glutamine	-3.5
	Aspartic Acid	-3.5
	Asparagine	-3.5
	Lysine	-3.9
	Arginine	-4.5
25		

Thus, for example, isoleucine, which has a
hydropathic index of +4.5, will preferably be exchanged
with an amino acid such as valine (+ 4.2) or leucine
(+ 3.8). Alternatively, at the other end of the scale,
lysine (- 3.9) will preferably be substituted for
arginine (-4.5), and so on.

Substitution of like amino acids may also be made on
the basis of hydrophilicity, particularly where the
biological functional equivalent protein or peptide
thereby created is intended for use in immunological

embodiments. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e. with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 \pm 1); glutamate (+3.0 \pm 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); (0 \pm 1); threonine (-0.4); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

Accordingly, these amino acid substitutions are generally based on the relative similarity of R-group substituents, for example, in terms of size, electrophilic character, charge, and the like. In general, preferred substitutions which take various of the foregoing characteristics into consideration include the following:

TABLE II

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>
5	Ala	gly; ser
	Arg	lys
	Asn	gln; his
	Asp	glu
	Cys	ser
10	Gln	asn
	Glu	asp
	Gly	ala
	His	asn; gln
	Ile	leu; val
15	Leu	ile; val
	Lys	arg; gln; glu
	Met	leu; ala
	Ser	thr
	Thr	ser
20	Trp	tyr
	Tyr	trp; phe
	Val	ile; leu

25 Preparation of Monoclonal Antibodies
 to *M. catarrhalis* OMPs

30 Monoclonal antibodies specific for the *Moraxella*
catarrhalis OMPs of the present invention may be prepared
 using conventional immunization techniques. Initially, a
 composition containing antigenic epitopes of the OMP,
 such as an outer membrane vesicle preparation, can be
 used to immunize an experimental animal, such as a mouse,
 from which a population of spleen or lymph cells are
 35 subsequently obtained. The spleen or lymph cells can
 then be fused with cell lines, such as human or mouse
 myeloma strains, to produce antibody-secreting

hybridomas. These hybridomas may be isolated to obtain individual clones which can then be screened for production of antibody to the desired OMP.

5 In particular aspects, the present invention utilizes outer membrane fragments from *M. catarrhalis* to induce an immune response in experimental animals. Following immunization, spleen cells are removed and fused, using a standard fusion protocol (see, e.g., The
10 Cold Spring Harbor Manual for Hybridoma Development, incorporated herein by reference) with plasmacytoma cells to produce hybridomas secreting monoclonal antibodies against outer membrane proteins. Hybridomas which
15 produce monoclonal antibodies to the selected OMP are identified using standard techniques, such as ELISA and Western blot methods.

 Hybridoma clones can then be cultured in liquid media and the culture supernatants purified to provide
20 the OMP-specific monoclonal antibodies.

Use of Monoclonal Antibodies to OMP Antigens

 In general, monoclonal antibodies to the desired OMP
25 antigen of *M. catarrhalis* can be used in both the diagnosis and treatment of *M. catarrhalis* infections.

 It is proposed that the monoclonal antibodies of the present invention will find useful application in
30 standard immunochemical procedures, such as ELISA and Western blot methods, as well as other procedure which may utilize antibody specific to OMP epitopes. These OMP-specific monoclonal antibodies are anticipated to be useful various ways for the treatment of *M. catarrhalis*
35 infections through, for example, their application in passive immunization procedures.

Additionally, it is proposed that monoclonal antibodies specific to the particular OMP may be utilized in other useful applications. For example, their use in immunoabsorbent protocols may be useful in purifying native or recombinant OMP species or variants thereof.

Studies have shown that antibody preparations against the OMP antigens of the invention have a significant protective effect against *M. catarrhalis* infection. The present inventors have shown that passive immunization with monoclonal antibodies specific for these OMPs significantly reduce the numbers of *M. catarrhalis* organisms following a bolus injection of bacteria. This demonstrates that these OMP antigens may be employed in making gammaglobulin preparations for use in passive immunization against disorders associated with *M. catarrhalis* infections, or used directly as vaccine components.

Recombinant Cloning Genes Encoding *M. catarrhalis* OMPs

To obtain suitable gammaglobulin preparations, one may desire to prepare monoclonal antibodies, preferably human or humanized hybridomas. Alternatively, it is proposed that one may desire to use globulin fractions from hyperimmunized individuals.

The present invention also involves isolating *M. catarrhalis* OMP genes, or sequence variants, incorporating DNA segments encoding the 30 kD, 80 kD or HMWP OMP gene into a suitable vector, and transforming a suitable host, such that recombinant proteins, or variants thereof, are expressed. It will be appreciated by those of skill in the art that in light of the present disclosure the invention is also applicable to the isolation and use of the OMP gene sequences from any suitable source that includes appropriate coding

sequences, such as any *M. catarrhalis* subspecies or isolate that expresses the desired OMP. Such sources may be readily identified by immunological screening with monoclonal antibodies to the selected OMP.

5

The preferred application of the present invention to the isolation and use of OMP-encoding DNA involves generally the steps of (1) isolation of *Moraxella* genomic DNA; (2) partial restriction enzyme digestion of the genomic DNA with an enzyme such as *Pst*I, (the selected restriction enzyme is not crucial) to provide DNA having an average length of, e.g., 6 to 23 kb; (3) ligation of the partially digested DNA into a selected site within a selected vector, such as pBR322 (again, other plasmid or phage vectors may be used at this step, as desired); (4) transformation, transfection or electroporation of suitable host cells, e.g., *E. coli* cells, with the recombinant vector; and (5) selection of colonies expressing the desired OMP through the application of specifically designed screening protocols. Following identification of a clone which contains the OMP gene, one may desire to reengineer the gene into a preferred host/vector/promoter system for enhanced production of the outer membrane protein, or sequence variants thereof.

25

Through application of the foregoing general steps, the inventors have succeeded in identifying and selecting a number of clones which contain *M. catarrhalis* OMP genes in a manner which allows it to produce the corresponding outer membrane protein.

30

In a preferred application of these techniques, genomic DNA from *Moraxella catarrhalis* strain 035E was isolated from bacteria through the use of SDS, ribonuclease and proteinase K treatment, phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Conditions were determined for achieving

35

an appropriate partial restriction enzyme digestion, such as would provide fragments on the order of 6-23 kb in length, using a restriction enzyme, such as *Pst*I. After size fractionation, the partially digested *Moraxella* DNA fragments of the selected size range were ligated with fully digested vector, such as pBR322, which was fully digested with *Pst*I to generate compatible sites for ligation with the genomic DNA fragments.

Following the ligation, the recombinant vectors are then used to transform a suitable host, such as *E. coli* RR1, to produce a recombinant library having members that express *M. catarrhalis* protein species encoded by the DNA fragment inserts. The recombinant microbial clones are cultivated, preferably on the surface of a nutrient agar, to form visible colonies. Those colonies expressing surface-exposed *M. catarrhalis* outer membrane proteins are then identified using monoclonal antibodies to *M. catarrhalis* OMPs in a colony blot radioimmunoassay. Recombinant *E. coli* clones expressing proteins having epitopes reactive with anti-OMP antibodies may then be cultured in desired quantities.

Host Cell Cultures and Vectors

In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and constructing the vectors useful in the invention. For example, *E. coli* strain RR1 is particularly useful. Other microbial strains which may be used include *E. coli* strains such as *E. coli* LE392, *E. coli* B, and *E. coli* X 1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes are also preferred for expression. The aforementioned strains, as well as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325), bacilli such as

Bacillus subtilis, or other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcescens*, and various *Pseudomonas* species may be used.

5 In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of
10 providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar et al., 1977). pBR322 contains genes for ampicillin and
15 tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins.

20 In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as a transforming vector in connection with these hosts. For example, the phage
lambda GEMTM-11 may be utilized in making recombinant
25 phage vector which can be used to transform host cells, such as *E. coli* LE392.

 Those promoters most commonly used in recombinant DNA construction include the B-lactamase (penicillinase)
30 and lactose promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) and a tryptophan (trp) promoter system (Goeddel et al., 1980; EPO Appl. Publ. No. 0036776). While these are the most commonly
35 used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker

to ligate them functionally with plasmid vectors (EPO Appl. Publ. No. 0036776).

5 In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is
10 commonly used (Stinchcomb et al., 1979; Kingsman et al., 1979; Tschemper et al., 1980). This plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1
15 (Jones, 1977). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

20 Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., 1980) or other glycolytic enzymes (Hess et al., 1968; Holland et al., 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase,
25 pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated
30 with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the
35 promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the

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aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7, 293 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated

with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

5 As origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host
10 cell chromosome, the latter is often sufficient.

Sequencing of OMP Genes

15 After cloning the gene encoding the selected OMP, one will desire to perform restriction mapping and DNA sequence analysis, e.g., by the dideoxy method of Sanger et al. (1977). Both the DNA and the deduced amino acid sequence can then be compared with known sequences to determine homologies with known proteins. The amino acid
20 sequence of the protein will reveal the nature of the various domains, e.g., cytoplasmic, membrane-spanning, and substrate binding domains, and give important information in terms of approaches to improving the structure of the enzyme through genetic engineering
25 techniques.

30 Through the use of computerized peptide sequence analysis program (DNASTar Software, DNASTar, Inc., Madison, Wisc.), particular hydrophilic peptidyl regions of the OMP antigen may be identified which are likely to constitute epitopic core sequences, comprising particular epitopes of the protein, as well as biologically functional equivalents of the foregoing peptides, as explained in more detail below.

Preparation of OMP Variants

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, derived from the OMP antigen sequence, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art as exemplified by publications (Adelman et al., 1983). As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (Messing et al., 1981). These phage are readily commercially available and their use is generally well known to those skilled in the art.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector which includes within its sequence a DNA sequence which encodes the OMP antigen. An

oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example by the method of Crea et al. (1978). This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected OMP gene using site-directed mutagenesis is provided as a means of producing potentially useful OMP species and is not meant to be limiting as there are other ways in which sequence variants of the OMP may be obtained. For example, recombinant vectors encoding the desired OMP gene may be treated with mutagenic agents to obtain sequence variants (see, e.g., a method described by Eichenlaub, 1979) for the mutagenesis of plasmid DNA using hydroxylamine.

25

Use of Nucleic Acid Sequences

As mentioned, in certain aspects, the DNA sequence information provided by the present disclosure allows for the preparation of relatively short DNA (or RNA) sequences having the ability to specifically hybridize to gene sequences of the selected OMP antigen gene. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of the natural sequence or derived from flanking regions of the OMP gene, such as regions downstream of the gene as found in plasmid pBR322. The ability of such nucleic acid

probes to specifically hybridize to OMP gene sequences
lend them particular utility in a variety of embodiments.
Most importantly, the probes can be used in a variety of
diagnostic assays for detecting the presence of
5 pathogenic organisms in a given sample. However, other
uses are envisioned, including the use of the sequence
information for the preparation of mutant species
primers, or primers for use in preparing other genetic
constructions.

10 To provide certain of the advantages in accordance
with the invention, the preferred nucleic acid sequence
employed for hybridization studies or assays includes
sequences that are complementary to at least a 10 to 20,
15 or so, nucleotide stretch of the sequence. A size of at
least 10 nucleotides in length helps to ensure that the
fragment will be of sufficient length to form a duplex
molecule that is both stable and selective. Molecules
having complementary sequences over stretches greater
20 than 10 bases in length are generally preferred, though,
in order to increase stability and selectivity of the
hybrid, and thereby improve the quality and degree of
specific hybrid molecules obtained. Thus, one will
generally prefer to design nucleic acid molecules having
25 OMP gene-complementary stretches of 15 to 20 nucleotides,
or even longer where desired. Such fragments may be
readily prepared by, for example, directly synthesizing
the fragment by chemical means, by application of nucleic
acid reproduction technology, such as the PCR technology
30 of U.S. Patent 4,603,102, or by introducing selected
sequences into recombinant vectors for recombinant
production.

35 In that the OMP antigens of the present invention
are believed to be indicative of pathogenic *Moraxella*
species, the present invention will find particular
utility as the basis for diagnostic hybridization assays

for detecting OMP-specific RNA or DNA in clinical samples. Exemplary clinical samples that can be used in the diagnosis of infections are thus any samples which could possibly include *Moraxella* nucleic acid, including
5 middle ear fluid, sputum, bronchoalveolar fluid, amniotic fluid or the like. A variety of hybridization techniques and systems are known which can be used in connection with the hybridization aspects of the invention, including diagnostic assays such as those described in
10 Falkow et al., U.S. Patent 4,358,535.

Accordingly, the nucleotide sequences of the invention are important for their ability to selectively form duplex molecules with complementary stretches of the
15 corresponding OMP genes. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one
20 will typically desire to employ relatively stringent conditions to form the hybrids, for example, one will select relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M NaCl at temperatures of 50°C to 70°C. These conditions are
25 particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, for example, where
30 one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template, less stringent hybridization conditions are called for in order to allow formation of the heteroduplex. In these circumstances, one would desire to employ conditions such
35 as 0.15M-0.9M salt, at temperatures ranging from 20°C to 55°C. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition

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of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, one may desire to employ nucleic acid probes to isolate variants from clone banks containing mutated clones. In particular embodiments, mutant clone colonies growing on solid media which contain variants of the OMP sequence could be identified on duplicate filters using hybridization conditions and methods, such as those used in colony blot assays, to only obtain hybridization between probes containing sequence variants and nucleic acid sequence variants contained in specific colonies. In this manner, small hybridization probes containing short variant sequences of the OMP gene may be utilized to identify those clones growing on solid media which contain sequence variants of the entire OMP gene. These clones can then be grown to obtain desired quantities of the variant OMP nucleic acid sequences or the corresponding OMP antigen.

In clinical diagnostic embodiments, nucleic acid sequences of the present invention are used in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred diagnostic embodiments, one will likely desire to employ an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophoto-

metrically, to identify specific hybridization with pathogen nucleic acid-containing samples.

5 In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridizations as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) from suspected clinical samples, such as exudates, body fluids (e.g.,
10 amniotic fluid, middle ear effusion, bronchoalveolar lavage fluid) or even tissues, is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired
15 conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the
20 hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

In other embodiments, it is proposed that OMP
25 sequences or variants thereof may be used to provide highly specific and sensitive detection of *M. catarrhalis* when used as reagents in polymerase chain reaction (PCR) assays. In general, by applying the PCR technology as set out, e.g., in U.S. Patent 4,60,102, one may utilize
30 various portions of the OMP sequence as oligonucleotide probes for the PCR amplification of a defined portion of OMP nucleic acid in a sample. The amplified portion of the OMP sequence may then be detected by hybridization with a hybridization probe containing a complementary
35 sequence. In this manner, extremely small concentrations of *M. catarrhalis* nucleic acid may be detected in a sample utilizing OMP sequences.

In other embodiments, OMP sequences may be utilized in PCR formats for the *in vitro* preparation of desired quantities of selected portions of the OMP gene. By
5 amplifying selected gene portions of a selected OMP gene and then incorporating those portions into vectors, one can also prepare recombinant clones which express OMP variants, including subfragments of the OMP antigen. In
10 this manner, peptides carrying antigen epitopes of the outer membrane protein may be prepared and utilized for various purposes.

Immunoassays

15 As noted, it is proposed that OMP peptides of the invention will find utility as immunogens, e.g., in connection with vaccine development, or as antigens in immunoassays for the detection of anti-OMP antigen-
20 reactive antibodies. Turning first to immunoassays, in their most simple and direct sense, preferred immunoassays of the invention include the various types of enzyme linked immunosorbent assays (ELISAs) known to
25 the art. However, it will be readily appreciated that the utility of OMP peptides is not limited to such assays, and that other useful embodiments include RIAs and other non-enzyme linked antibody binding assays or procedures.

In the preferred ELISA assay, peptides incorporating
30 OMP antigen sequences are immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, one will desire to bind or coat a nonspecific
35 protein such as bovine serum albumin (BSA) or casein onto the well that is known to be antigenically neutral with regard to the test antisera. This allows for blocking of

nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

5 After binding of antigenic material to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the antisera or clinical or biological extract to be tested in a manner
10 conducive to immune complex (antigen/antibody) formation. Such conditions preferably include diluting the antisera with diluents such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of
15 nonspecific background. The layered antisera is then allowed to incubate for from 2 to 4 hours, at temperatures preferably on the order of 25° to 27°C. Following incubation, the antisera-contacted surface is washed so as to remove non-immunocomplexed material. A
20 preferred washing procedure includes washing with a solution such as PBS/Tween, or borate buffer.

 Following formation of specific immunocomplexes between the test sample and the bound antigen, and
25 subsequent washing, the occurrence and even amount of immunocomplex formation may be determined by subjecting same to a second antibody having specificity for the first. Of course, in that the test sample will typically be of human origin, the second antibody will preferably
30 be an antibody having specificity in general for human IgG. To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example,
35 one will desire to contact and incubate the antisera-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions

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which favor the development of immunocomplex formation (e.g., incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

5 After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-
10 sulfonic acid [ABTS] and H_2O_2 , in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, e.g., using a visible spectra spectrophotometer.

15 Vaccine Preparation and Use

 Immunogenic compositions, proposed to be suitable for use as a vaccine, may be prepared most readily directly from immunogenic OMP proteins and/or peptides
20 prepared in a manner disclosed herein. Preferably the antigenic material is extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle.

25 The preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference.
30 Typically, such vaccines are prepared as injectables. Either as liquid solutions or suspensions: solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may
35 also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient.

Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides: such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%.

The proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic

bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

5 The vaccines are administered in a manner compatible
with the dosage formulation, and in such amount as will
be therapeutically effective and immunogenic. The
quantity to be administered depends on the subject to be
treated, including, e.g., the capacity of the
10 individual's immune system to synthesize antibodies, and
the degree of protection desired. Precise amounts of
active ingredient required to be administered depend on
the judgment of the practitioner. However, suitable
dosage ranges are of the order of several hundred
micrograms active ingredient per vaccination. Suitable
15 regimes for initial administration and booster shots are
also variable, but are typified by an initial
administration followed by subsequent inoculations or
other administrations.

20 The manner of application may be varied widely. Any
of the conventional methods for administration of a
vaccine are applicable. These are believed to include
oral application on a solid physiologically acceptable
base or in a physiologically acceptable dispersion,
25 parenterally, by injection or the like. The dosage of
the vaccine will depend on the route of administration
and will vary according to the size of the host.

30 Various methods of achieving adjuvant effect for the
vaccine includes use of agents such as aluminum hydroxide
or phosphate (alum), commonly used as 0.05 to 0.1 percent
solution in phosphate buffered saline, admixture with
synthetic polymers of sugars (Carbopol) used as 0.25
percent solution, aggregation of the protein in the
35 vaccine by heat treatment with temperatures ranging
between 70° to 101°C for 30 second to 2 minute periods
respectively. Aggregation by reactivating with pepsin

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5 treated (Fab) antibodies to albumin, mixture with
bacterial cells such as C. parvum or endotoxins or
lipopolysaccharide components of gram-negative bacteria,
emulsion in physiologically acceptable oil vehicles such
as mannide mono-oleate (Aracel A) or emulsion with 20
percent solution of a perfluorocarbon (Fluosol-DA) used
as a block substitute may also be employed.

10 In many instances, it will be desirable to have
multiple administrations of the vaccine, usually not
exceeding six vaccinations, more usually not exceeding
four vaccinations and preferably one or more, usually at
least about three vaccinations. The vaccinations will
normally be at from two to twelve week intervals, more
15 usually from three to five week intervals. Periodic
boosters at intervals of 1-5 years, usually three years,
will be desirable to maintain protective levels of the
antibodies. The course of the immunization may be
followed by assays for antibodies for the supernatant
20 antigens. The assays may be performed by labeling with
conventional labels, such as radionuclides, enzymes,
fluorescers, and the like. These techniques are well
known and may be found in a wide variety of patents, such
as U.S. Patent Nos. 3,791,932; 4,174,384 and 3,949,064,
25 as illustrative of these types of assays.

EXAMPLE I

EDTA-BASED EXTRACTION OF OUTER MEMBRANE FRAGMENTS

30 In order to obtain antibody to the OMP antigens,
outer membrane fragments from *M. catarrhalis* strain 035E
were prepared as an immunogen. *M. catarrhalis* strain
035E cells were grown on agar plates using brain heart
35 infusion broth. Plates were incubated at 37° C in a
candle extinction jar. Outer membrane fragments were
subsequently prepared from these cells by the EDTA-based

extraction procedure of Murphy et al., *Microb. Path.*,
1989.

EXAMPLE II

5

ISOLATION OF *M. CATARRHALIS* OMPs

In light of the present disclosure's identification
of monoclonal antibodies specific to selected *M.*
10 *catarrhalis* OMPs, it is proposed that the corresponding
OMP antigen may be purified using the following general
procedure. Cell envelopes will be prepared by sonication
or outer membrane fragments will be extracted by EDTA-
based treatment of whole *M. catarrhalis* cells. These
15 membranes will be treated with ionic or non-ionic
detergents to release the desired proteins which can then
be purified by using conventional column chromatography
or by immunoaffinity techniques.

20

EXAMPLE III

PREPARATION OF MONOCLONAL ANTIBODIES SPECIFIC FOR *M. CATARRHALIS* OUTER MEMBRANE PROTEINS

25 The present example illustrates the steps employed
by the inventors in reducing certain aspects of the
invention to practice. In particular, this example
relates to the generation and identification of
hybridomas that produce monoclonal antibodies to the 30
kD, 80 kD or HMWP OMP antigen. Once hybridomas secreting
monoclonal antibodies to surface-exposed OMP antigens
from *M. catarrhalis* were identified, those determined to
produce antibody to these OMP antigens were selected and
cultured to produce antibody for use in other studies,
35 such as those involving pulmonary clearance of *M.*
catarrhalis.

BALB/c mice were immunized by intraperitoneal injection with outer membrane fragments of *M. catarrhalis* strain 035E prepared by the EDTA-based extraction procedure. Each animal was immunized with 50-100 μ g protein in 0.1 ml of Freund's complete adjuvant. One month later, the animals were boosted with an identical quantity of this same protein preparation in incomplete Freund's adjuvant. Three weeks later, the mice were given an intravenous injection (into the tail vein) with 50 μ g protein of the same membrane preparation suspended in PBS.

The "pancake" fusion method was employed as follows:

SP₂₀-Ag14 plasmacytoma cells were employed. These cells were maintained in DMEM (Dulbecco's Modified Eagle Medium)/ Penicillin-Streptomycin-Glutamine with 15% fetal bovine serum, 1% Fungizone and 8-azaguanine. Two weeks prior to the fusion, some of the cells were split into media with 1% Fungizone but lacking 8-azaguanine. These cells were maintained for 10 days at a density of no greater than $1-2 \times 10^5$ /ml. Beginning three days before the fusion, SP₂₀ cells were subcultured every 24 hours and maintained at an approximate density of $2-3 \times 10^5$ /ml. Three days before the fusion, the mice were boosted intravenously with about 50 μ g of protein immunogen. On the day of the fusion, two mice were sacrificed by cervical dislocation.

The spleens were removed aseptically and macerated. Spleen cells were collected in 10 mls of DMEM-HY media (60 ml NCTC-109, 6 tubes hypoxanthine-thymidine-glycine stock soln., 6 tubes oxaloacetic acid-bovine insulin stock soln., 12 ml penicillin-streptomycin-glutamine, 2.7 ml 100mM Na pyruvate, and 508 ml DMEM). At room temperature, SP₂₀ cells and spleen cells were collected by centrifugation at $170 \times g$ for 11 min. in their respective

tubes. SP₂₀ cells and spleen cells were each resuspended in a total of 5 mls of DMEM-HY media.

5 The hypoxanthine-thymidine-glycine stock solution was prepared by adding 136 mg hypoxanthine to 100 ml 0.1 M HCl, 38.7 mg thymidine to 100 ml H₂O, and 2.3 mg glycine to 20 ml H₂O. These solutions were dissolved separately, combined and then aliquoted into 2.2 ml volumes.

10 The oxaloacetic acid-bovine insulin stock solution was prepared by dissolving 80.3 mg bovine insulin in 100 ml H₂O, adding 1.32 gm oxaloacetic acid and aliquoting into 1 ml. volumes.

15 Spleen cells were then diluted to 2×10^8 cells/5 mls and the SP₂₀ cells was diluted to 2×10^7 cells/5 mls. The ratio of spleen cells to SP₂₀ cells was 10:1. Spleen cells were then mixed with SP₂₀ cells in a ratio of 1:1. The spleen-SP₂₀ mixture was then treated with 3 mls of 50%
20 PEG/DMEM-HY media for 35 sec. Fused spleen-SP₂₀ cells were washed immediately with DMEM-HY and incubated in 30% HY:HIFCS (35 ml DMEM-HY, 15 ml FBS, filter) for 24 hours at 37°C. 24 hours after the fusion, media and fused cells were collected in 20% HY:HIFCS (80 ml DMEM-HY, 20
25 ml FBS, filter) by centrifugation at 170 x g for 5 min. The fused cells were then resuspended in 100 mls of 20% HAT:HIFCS and transferred to 96-well microtiter plates, 100 µl/well. One week after the fusion, 100 µl of 20%
30 HY:HIFCS were added to each well. Two weeks after the fusion, when wells containing proliferating hybrid cells became acidic, each positive well was split into a 2 ml well on a 24-well plate and the culture supernatant assayed for antibody characterization.

35 Supernatants from these clones were screened for antibodies to *M. catarrhalis* by ELISA binding and Western blot methods using EDTA-extracted outer membrane

fragments of *M. catarrhalis* strain 035E as antigen for the ELISA, and whole cell lysates of this strain as antigen for Western blots. Positive supernatants were then tested by the indirect antibody accessibility RIA to
5 investigate the surface exposure of outer membrane antigens as described by Kimura et al. (1985 and 1986).

Positive hybridomas were then cultured in standard DME and the monoclonal antibodies were purified from
10 culture supernatants on Protein A - Sepharose CL-4B as described by Ey et al., 1978.

Each Mab identified as being reactive with *M. catarrhalis* in Western blot analysis was used in the
15 indirect antibody accessibility assay to determine if these Mabs were reactive with surface-exposed determinants of this organism. The antibody accessibility assay performed was described by Patrick et al., 1987.

20 Mab 10F3, which reacted with an antigen with an apparent MW of approximately 80,000 in Western blot analysis, was shown to bind to the surface of whole cells of strain 035E. This Mab reacted with 4 of 10 different
25 *M. catarrhalis* strains tested in colony blot-RIA analysis by the method of Gulig et al., 1987.

Mab 17C7 reacted with two different size bands in Western blot analysis. This Mab reacted with a band near
30 the top of the gel that migrated in a diffuse form and sometimes with a second band that migrated with an apparent MW of between about 200 and about 700 kD. For the purpose of clarity, the Mab will be defined as being reactive with the "HMWP" antigen. This Mab bound to the
35 surface of strain 035E and reacted with all ten different *M. catarrhalis* strains tested in the colony blot RIA.

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Mab 8B6 reacted with an antigen with an apparent MW of approximately 30,000 in Western blot analysis. This Mab was also reactive with the surface of strain 035E and reacted with all ten different *M. catarrhalis* strains tested in the colony blot-RIA.

EXAMPLE IV

PULMONARY CLEARANCE OF *M. CATARRHALIS* USING MONOCLONAL ANTIBODIES SPECIFIC FOR THE 30, 80, AND 100 KD OMPs

The present example illustrates steps employed by the inventors in reducing certain aspects of the invention to practice. This example demonstrates the ability of monoclonal antibodies to the 30 kD, 80 kD and HMWP OMPs to enhance pulmonary clearance of *M. catarrhalis* using a murine model system. Thus, this example demonstrates that antibodies to the 30 kD, 80 kD or HMWP OMP may be useful for passive immunization and that vaccines comprising these OMPs are likely to provide active immunity against *M. catarrhalis* infections.

A. Antibody Administration

Eighteen hours prior to bacterial challenge, groups of 5 mice were passively immunized by intravenous administration of monoclonal antibody 17C7, 8B6 or 10F3. Control animals were immunized with an irrelevant antibody, 2H11, which was directed against an outer membrane protein of *Haemophilus ducreyi*. Each animal received an equivalent amount of purified antibody corresponding to 150 μ g of total protein.

B. Method of Bacterial Inoculation

Mice were anaesthetized by intramuscular injection of 2 mg of ketamine HCL (Fort Dodge Lab, Fort Dodge, IA)

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and 0.2 mg of acepromazine maleate (Fort Dodge Lab). After tracheal exposure each animal was intubated transorally with a 20 gauge intravenous catheter which was advanced until it could be visualized through the translucent tracheal wall. A PE-10 polyethylene tube containing 5 μ l of bacterial suspension was then passed through the catheter into the lung where the bacteria were deposited with 150 μ l of air. This technique delivered the inoculum to a localized, peripheral segment of the lung. In all experiments, mice were challenged with *M. catarrhalis* strain 035E.

C. Pulmonary Clearance

In each experiment, 5 mice were sacrificed by intraperitoneal injection of 0.75 mg of sodium pentobarbital (Abbott Labs, Chicago, IL) immediately after inoculation (0 h), to determine bacterial deposition in the lungs. At 6 hours after challenge, experimental (17C7-, 8B6- or 10F3-immunized) and control (2H11 immunized) groups were sacrificed, and the number of viable bacteria remaining in the lungs was determined as follows: the lungs from each animal were removed aseptically and homogenized in 2 ml of sterile BHI broth in a tissue homogenizer followed by grinding in a tissue grinder until smooth. The homogenate was serially diluted in BHI broth, plated on BHI agar and incubated at 37°C in an air incubator with a 5% CO₂ atmosphere for 24 h. Clearance of *M. catarrhalis* from the lungs is expressed as the percentage of colony forming units (cfu) remaining in the lung at each time point compared with the mean cfu of bacteria present at 0 h in the same experiment.

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RESULTS

5 The mean number of viable bacteria remaining in the lungs of immunized and control mice after bolus deposition of 0.98×10^5 to 2.0×10^5 cfu of *M. catarrhalis* 035E was determined and expressed as a percentage of the initial inoculum.

TABLE III

15	Immunization Regimen	% of Bacteria Remaining at 6 h Post-Challenge	
		Expt. #1	#2
20	No immunization	134	109
	2H11 immunization	113	108
	17C7 immunization	27	22
	10F3 immunization	10	13

25
30
35 It will be noted that Table III does not include pulmonary clearance data for Mab 8B6. This Mab initially appeared positive, and a further study failed to duplicate this earlier positive finding as to pulmonary clearance. However, in follow-up studies it appears as though Mab 8B6 has at most a limited protective effect, but is not as protective as 17C7 or 10F3. In these follow-up studies (two experiments), 8B6 exhibited an average for % bacteria remaining at 6 hours of 38 versus about 97 for the control Mab, 2H11.

EXAMPLE V

CLONING THE GENE ENCODING THE
80 KD OMP (10F3-REACTIVE) FROM *M. CATARRHALIS*

5

The present example illustrates steps employed by the inventor in cloning the gene encoding for the 80 kD OMP from *M. catarrhalis*. The present example discloses one or more preferred recombinant *E. coli* clones, expressing the 80 kD OMP antigen, isolated by the following procedures.

10

A. Isolation of genomic DNA

15

M. catarrhalis strain 035E was used as a representative *Moraxella* pathogen in this study. Genomic DNA from *M. catarrhalis* strain 035E was extracted and purified as follows. *M. catarrhalis* cells (approximately 2 gms wet weight) were scraped from agar plates and resuspended in 20 mls. PBS. To this suspension was added 3.2 ml 10% (w/v) SDS and 1 ml RNase (10 mg/ml). This mixture was incubated at 37°C, then 3 mg proteinase K added, followed by further incubation at 55°C overnight. The incubated mixture was then extracted once with phenol, twice with phenol:chloroform:isoamyl alcohol, and three times with chloroform:isoamyl alcohol. The resulting DNA was then precipitated with two volumes of absolute ethanol, and collected with a Pasteur pipet.

20

25

B. Preparation of an *M. catarrhalis* genomic library in pBR322

30

The partial digestion of genomic DNA was achieved by incubating 100 µg portions of *M. catarrhalis* genomic DNA with varying amounts of the restriction enzyme *Pst*I in a reaction volume of about 1.5 ml. at 37°C. for 1 hr. The partially digested genomic DNA was then size fractionated

35

by sucrose density gradient centrifugation. Fractions containing DNA fragments from about 6 kb to 23 kb in length were selected and dialyzed to obtain purified genomic DNA fragments for ligation with pBR322.

5

The plasmid vector pBR322 was fully digested with *Pst*I by incubating 15 μ g portions of pBR322 with 50 units of *Pst*I in a 100 μ l reaction volume at 37°C. for 18 hrs. Ligation of the purified DNA fragments into the *Pst*I-digested pBR322 vector was accomplished by incubating 300 ng of the purified DNA fragments and *Pst*I-digested pBR322 together with ATP and T4 DNA ligase under conditions described by Maniatis et al. (1982). After ligation, the DNA was diluted 1:5 with 10 mM TRIS-HCl (pH 8.0) and was used to transform *E. coli* RR1 made competent by the CaCl₂ method.

10

15

C. Screening transformed RR1 colonies by colony blot-radioimmunoassay for *M. catarrhalis* OMP expression

20

A colony blot RIA was then carried out as described by Gulig et al. (1987) with monoclonal antibody 10F3 as the primary antibody.

25

D. Characterizing recombinant *E. coli* clones expressing *M. catarrhalis* OMP antigens

Clones which reacted with monoclonal antibody 10F3 in the colony blot RIA were cultured using LB medium containing the antibiotic tetracycline (15 μ g/ml). Whole cell lysates of recombinant *E. coli* RR1 expressing *M. catarrhalis* OMP antigens were prepared as described by Patrick et al., 1987. Briefly, portions of these whole-cell lysates were subjected to SDS-PAGE as described in Gulig et al., 1987, and then stained with Coomassie blue

30

35

or transferred to nitrocellulose for Western blot analysis.

5 The results shown in Figure 1 indicate that a recombinant 80 kD OMP gene was expressed in the clone identified by monoclonal antibody 10F3. This clone has been subsequently designated pMEH100. For sequence analysis, a 2.5 kb subfragment of pMEH100 was subcloned in a pBluescript SK+ vector (pMEH120). A preliminary
10 restriction analysis of pMEH120 is shown in Figure 2.

EXAMPLE VI

15 CLONING OF THE GENES ENCODING THE 30 kD (8B6-REACTIVE) AND 100kD (17C7-REACTIVE) OUTER MEMBRANE PROTEINS OF *M. CATARRHALIS*

A. Isolation of genomic DNA

20 In other cloning procedures, *M. catarrhalis* genomic DNA was isolated and purified from strain 035E, and 100 μ g samples were partially digested with *Sau*3A (Promega Biotech) at room temperature, as described above for *Pst*I.

25

B. Preparation of a *M. catarrhalis* genomic DNA library using the bacteriophage vector λ GEM-11

30 The digested DNA was size-fractionated in sucrose density gradients and fragments of DNA 15 kb and larger were collected for use in library construction. These DNA fragments (1 μ g) were filled in using the Klenow procedure (Promega) at 14°C for 90 min. These fragments were then cleaned by standard procedures and ligated onto
35 the phage DNA arms and packaged using the protocol and reagents supplied by Promega in the LambdaGEM - 11* Xho I Half-Site Arms Cloning System, except that T4 DNA ligase

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from BRL was used. After packaging, the phage-based library was titered using E. coli LE392. This genomic library contained 50,000 recombinant clones.

5 C. Screening of the bacteriophage-based genomic
DNA library with monoclonal antibodies 17C7 and
 8B6

10 To screen the clone bank, 20,000 plaques were
immunoreacted with Mabs 17C7 and 8B6 using the plaque
screening procedure described in Current Protocols in
Molecular Biology (Wiley Interscience) using
radioiodinated goat anti-mouse Ig as the probe to detect
15 Mabs bound to plaque material. One recombinant phage
reactive with each Mab was ultimately identified.

 D. Characterization of the recombinant phages
 reactive with Mabs 17C7 and 8B6

20 Liquid lysate cultures of these recombinant phage
were prepared by the standard methods described in
Current Protocols in Molecular Biology. The DNA was
extracted using standard methods. Phage harvested from
liquid lysates were heated at 100°C for 3 min. in
25 standard SDS digestion buffer and then used for SDS-PAGE
and Western blot analysis to confirm that these
recombinant phage were expressing the appropriate *M.*
catarrhalis antigens.

30 The recombinant phage reactive with Mab 17C7,
designated MEH200, were found to comprise DNA coding for
the HMWP. A preliminary restriction map of MEH200 is
shown in Figure 3 to comprise an insert of about 11 kb in
size. A second clone, designated pMEH3000, was found to
35 incorporate a DNA segment of about 18 kb in size,
encoding for the Mab 8B6-reactive 30 kD antigen.

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Figure 4 is an illustrative Western blot analysis of proteins from E. coli clone LE392/8B6, which expresses the 30 kD OMP antigen. In this study, the various indicated samples were subjected to PAGE, transferred to a nitrocellulose membrane, and probed with the 30 kD OMP-specific monoclonal antibody 8B6. As can be seen, a band having an approximate molecular weight of 30 kD is seen in the LE 392/8B6 lane (lane C), and a similar band is seen in the position control lane (lane F). The nature of the two additional bands seen in the LE 392/8B6 lane (lane C) is unclear, but they could be due to processing of the recombinant protein or overloading of the gel. The bands seen in the negative control lanes (lanes D and E) are clearly due to spillover from lanes C and F.

Figure 5 shows a similar Western blot analysis of a phage lysate from a clone expressing the HMWP OMP, designated LE 392/17C7, probed with monoclonal antibody 17C7. Lanes C-E comprise phage lysate proteins from clone LE392/17C7. These lanes exhibit slight reactivity in the very high molecular range.

* * *

The present invention has been described in terms of particular embodiments found or proposed by the present inventors to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting in kind or amount of the biological

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action. All such modifications are intended to be included within the scope of the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

10

Adelman et al. (1983) *DNA* 2:183

15

Bolivar et al. (1977) *Gene* 2:95

Campagnari et al. (1987), *Infect. Immun.*, 55:882-7

Chang et al. (1978) *Nature* 375:615

20

Consensus (1989), *Pediatr. Infect. Dis. J.*, 8:S94-S97

Crea et al. (1978), *Proc. Natl. Acad. Sci. U.S.A* 75:5765

25

Eichenlaub, R. (1979) *J. Bacteriol* 138:559-566

EPO Appl. Publ. No. 0036776

Ey et al. (1978), *Immunochem* 5:429-436

30

Fiers et al. (1978) *Nature* 273:113

Goeddel et al. (1979) *Nature* 281:544

35

Goeddel et al. (1980) *Nucleic Acids Res.* 8:4057

Goldblatt et al. (1990), *Jrnl. Infect. Dis.*, 162:1128-1135

- Gulig et al. (1987) *Infect. Immun.* 55:513-520
- Hess et al. (1968) *J. Adv. Enzyme Reg.* 7:149
- 5 Hitzeman et al. (1980) *J. Biol. Chem.* 255:2073
- Holland et al. (1978) *Biochemistry* 17:4900
- Itakura et al. (1977) *Science* 198:1056
- 10 Jones (1977) *Genetics* 85:12
- Kimura et al. (1986) *Infect. Immun.* 50:69-79
- 15 Kimura et al. (1985) *Infect. Immun.* 47:253-259
- Kingsman et al. (1979) *Gene* 7:141
- Kyte et al. (1982) *J. Mol. Biol.* 157:105-132.
- 20 Maniatis et al. (1982) Molecular cloning: a laboratory manual Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- 25 Messing et al. Third Cleveland Symposium on Macromolecules and Recombinant DNA, Editor A. Walton, Elsevier, Amsterdam (1981)
- Murphy (1989), *Pediat. Infect. Dis. J.*, 8: S75-S77
- 30 Murphy et al. (1989), *Microb. Path.*, 6:159-174
- Murphy et al. (1990), *Am. Jrnl. Med.*, 88:5A-41S-5A-45S
- 35 Patrick et al. (1987), *Infect. Immun.*, 55:2902-2911

Sanger et al. (1977) *Proc. Natl. Acad. Sci. USA* 74:5463-5467

Stinchcomb et al. (1979) *Nature* 282:39

5

Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)

Tschemper et al. (1980) *Gene* 10:157

CLAIMS

1. An antigen composition comprising a purified protein or peptide antigen incorporating an epitope that is immunologically cross-reactive with *M. catarrhalis* 30 kD, 80 kD or HMWP OMP.
2. The composition of claim 1, wherein the antigen is further defined as the *M. catarrhalis* 30 kD, 80 kD or HMWP kD OMP.
3. The composition of claim 1, wherein the antigen is essentially free of antigenic epitopes from other *M. catarrhalis* antigens.
4. The composition of claim 1, wherein the antigen is further defined as a peptide incorporating such an epitope.
5. The composition of claim 4, wherein the antigen comprises a peptide of from 15 to 50 amino acids in length.
6. The composition of claim 5, wherein the antigen comprises a peptide of from 15 to 30 amino acids in length.
7. The composition of claim 4, wherein the peptide antigen incorporates an epitope of the *M. catarrhalis* 30 kD, 80 kD or HMWP OMP.

8. A process for preparing a composition in accordance with claim 1, comprising the steps of:

- 5 a) selecting cells capable of expressing a protein or peptide antigen incorporating an epitope that is immunologically cross-reactive with *M. catarrhalis* 30 kD, 80 kD or HMWP OMP;
- 10 b) culturing the cells under conditions effective to allow expression of the antigen; and
- c) collecting the antigen to prepare the composition.
- 15

9. The process of claim 8, wherein the antigen comprises the 30 kD, 80 kD or HMWP OMP of *M. catarrhalis*.

20 10. The process of claim 9 wherein the cells comprise *M. catarrhalis* cells.

25 11. The process of claim b, wherein the cells comprise recombinant host cells that express a recombinant DNA segment encoding the antigen.

30 12. The process of claim 11 wherein the recombinant host cells comprise bacterial host cells.

35 13. The process of claim 12, wherein the bacterial host cells comprise *E. coli*, *H. influenzae*, *Salmonella*, *Mycobacterium*, or *Bacillus subtilis* cells.

14. The process of claim 11, wherein the recombinant DNA segment encodes the 30 kD, 80 kD or HMWP OMP of *M. catarrhalis*, or an antigenic subfragment thereof.

5

15. The process of claim 8, further comprising purifying the antigen by a method that includes detergent extraction of outer membrane vesicles of *M. catarrhalis*.

10

16. An antigen composition prepared by a process as set forth in claim 8.

15

17. A DNA segment encoding a protein or peptide antigen incorporating an epitope that is immunologically cross-reactive with *Moraxella catarrhalis* 30 kD, 80 kD or HMWP OMP.

20

18. The DNA segment at claim 17, further defined as encoding the *Moraxella catarrhalis* 30 kD, 80 kD or HMWP OMP.

25

19. The DNA segment of claim 17, further defined as encoding a peptide incorporating such an epitope.

30

20. The DNA segment of claim 19, wherein the encoded peptide is from 15 to 50 amino acids in length.

35

21. A recombinant vector incorporating a DNA segment in accordance with claim 17.

22. A recombinant host cell comprising a DNA segment in accordance with claim 17.

5

23. The host cell of claim 22, wherein the DNA segment is introduced into the cell by means of a recombinant vector.

10

24. The host cell of claim 23, wherein the host cell is capable of expressing the DNA segment to produce the antigen.

15

25. The host cell of claim 24, further defined as capable of expressing the 30 kD, 80 kD or HMWP OMP of *Moraxella catarrhalis*.

20

26. The host cell of claim 25, further defined as capable of overexpressing the 80 kD OMP in relation to *Moraxella catarrhalis* cells.

25

27. An antibody to an antigen of claim 1.

30

28. The antibody of claim 27, further defined as a monoclonal antibody.

35

29. The antibody of claim 28, further defined as a monoclonal antibody that is cross-reactive with the same antigen as monoclonal antibody 10F3.

30. The antibody of claim 28, further defined as a monoclonal antibody that is cross-reactive with the same antigen as monoclonal antibody 17C7.

5

31. The antibody of claim 28, further defined as a monoclonal antibody that is cross-reactive with the same antigen as monoclonal antibody 8B6.

10

32. A process for detecting an antigen in accordance with claim 1 in a sample, comprising the steps of:

15

a) obtaining a sample suspected of containing such an antigen;

b) contacting the sample with an antibody as defined by claim 27 under conditions effective to allow the antibody to form an immunocomplex with antigen that may be present in the sample; and

20

c) detecting the presence of the antigen in the sample by detecting the formation of such an immunocomplex.

25

33. A process for detecting an antibody in accordance with claim 27 in a sample comprising the steps of:

30

a) obtaining a sample suspected of containing such an antibody,

35

b) contacting the sample with an antigen in accordance with claim 1 under conditions effective to allow the antigen to form an immunocomplex with antibody that may be present in the sample; and

- c) detecting the presence of such an antibody in the sample by detecting the formation of such an immunocomplex.

5

34. A kit for use in detecting the presence of an antigen in accordance with claim 1 in a sample, the kit comprising:

10

- a) an antibody in accordance with claim 27;
- b) an immunodetection reagent; and
- c) means for containing said antibody and reagent.

15

35. A kit for use in detecting the presence of an antibody in accordance with claim 27 in a sample, the kit comprising:

20

- a) an antigen in accordance with claim 1;
- b) an immunodetection reagent; and
- c) means for containing said antibody and reagent.

25

36. A method for inducing tolerance to *Moraxella catarrhalis* challenge in an animal, comprising providing to the animal an effective amount of an antibody in accordance with claim 27.

30

37. The method of claim 36, wherein the antibody is provided to the animal by means of passive immunotherapy, wherein such an antibody is introduced into the bloodstream of the mammal in effective amounts.

35

38. The method of claim 36, wherein the antibody is one
produced by the animal's own immune system and provided
5 to the mammal by means of immunization using an antigen
in accordance with claim 1.

39. A vaccine composition comprising an antigen in
10 accordance with claim 1, together with a pharmaceutically
acceptable carrier, diluent or adjuvant.

40. A pharmaceutical composition comprising an antibody
15 in accordance with claim 27, together with a
pharmaceutically acceptable carrier, diluent or adjuvant.

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FIG. 1

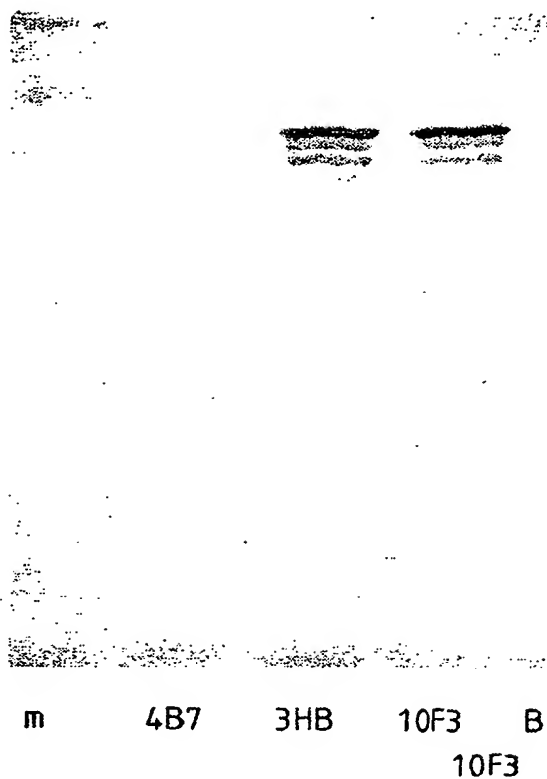
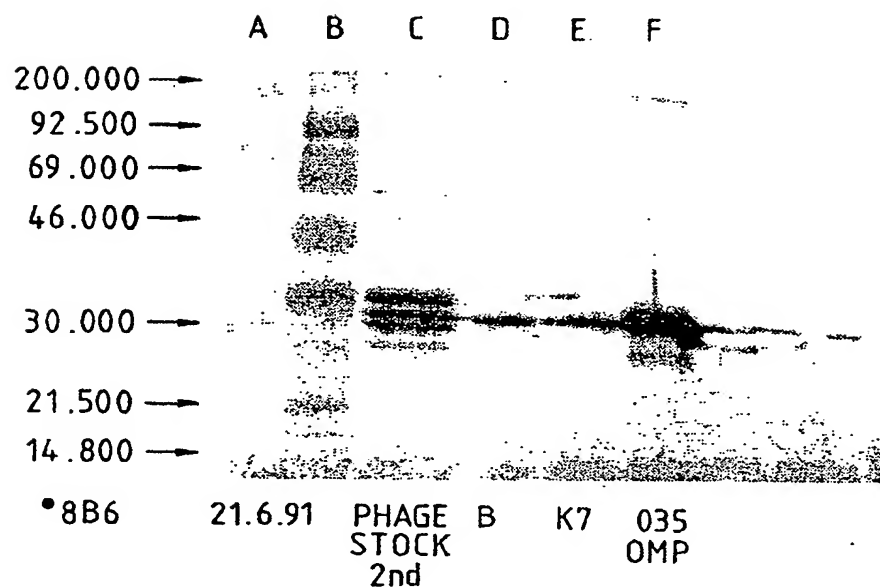
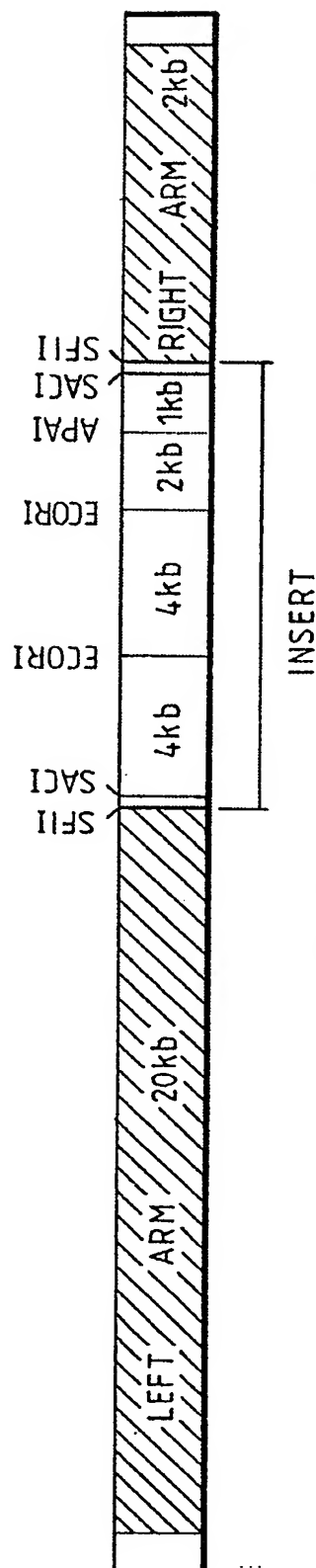
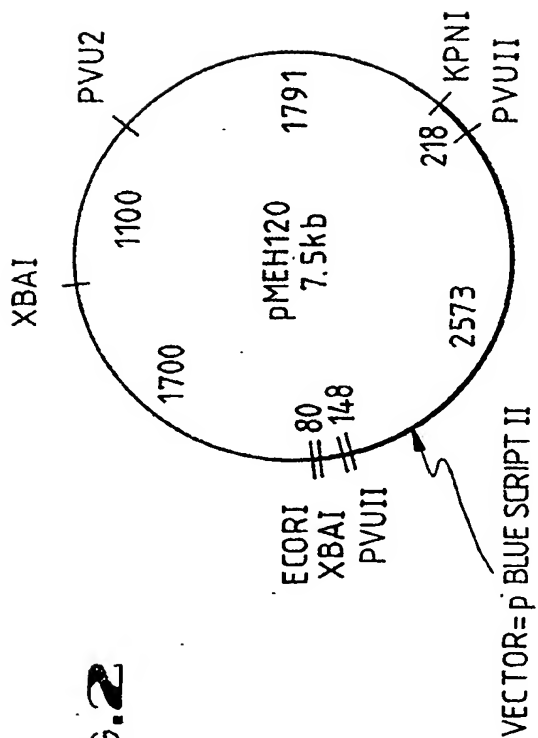


FIG. 4



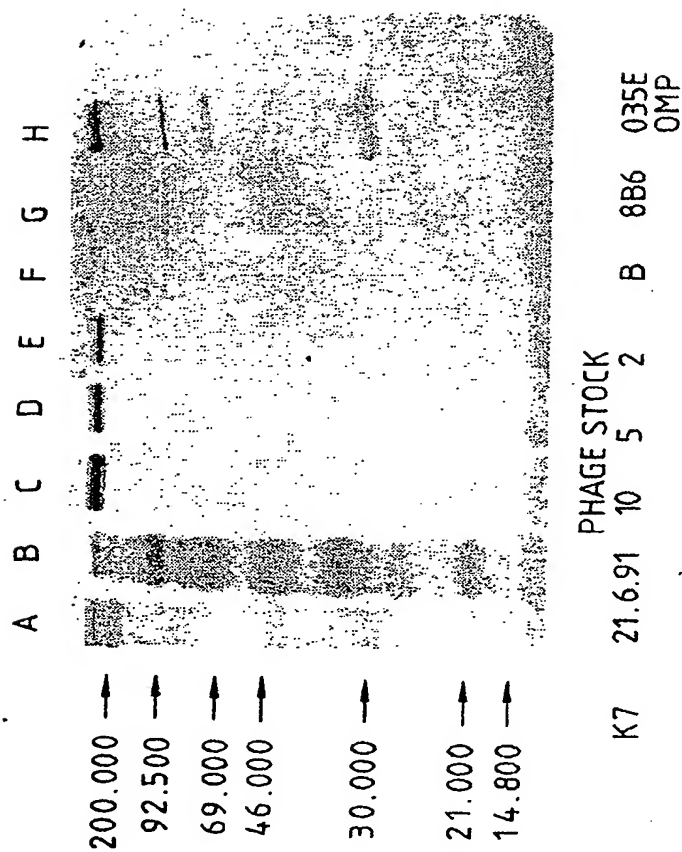
2/3



MEH200
VECTOR = LAMBDA GEM[®]-11 (PROMEGA)

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FIG. 5



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/06869

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K39/095;	C12N15/31;	C12P21/08;	C07K15/00
G01N33/569;	G01N33/577;	A61K37/02;	A61K39/395

II. FIELDS SEARCHEDMinimum Documentation Searched⁷

Classification System	Classification Symbols
Int.Cl. 5	C07K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸**III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹**

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P, X	<p>ABSTRACTS OF THE 1991 ICAAC, ABSTRACT NO. 278 page 145 M. HELMINEN ET AL. 'MOLECULAR CLONING OF A GENE ENCODING A SURFACE-EXPOSED OUTER MEMBRANE PROTEIN OF MORAXELLA-CATARRHALIS' & 'THIRTY-FIRST INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, CHICAGO, ILLINOIS, USA, SEPTEMBER 29-OCTOBER 2, 1991' see the whole document</p> <p style="text-align: center;">--- -/-</p>	17-19, 21-29, 32, 34

¹⁰ Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 17 NOVEMBER 1992	Date of Mailing of this International Search Report 27. 11. 92
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer THIELE U.H.-C.H. <i>L.H.</i>

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages.	Relevant to Claim No.
A	JOURNAL OF INFECTIOUS DISEASES vol. 158, no. 4, October 1988, CHICAGO, USA pages 761 - 765 L. C. BARTOS ET T. F. MURPHY 'Comparison of the Outer Membrane Proteins of 50 Strains of Branhamella catarrhalis' cited in the application see abstract ---	1,2
A	INFECTION AND IMMUNITY vol. 57, no. 10, October 1989, WASHINGTON US pages 2938 - 2941 T. F. MURPHY ET L. C. BARTOS 'Surface-Exposed and Antigenically Conserved Determinants of Outer Membrane Proteins of Branhamella catarrhalis' see abstract -----	39

INTERNATIONAL SEARCH REPORT

I. International application No.

PCT/US 92/ 06869

Box I— Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 36-38 are directed to a method of treatment of the animal body (see PCT-Rule 39.1(iv)) the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.